Appendix II

Denaturation and Change in Hydrogen-ion Binding.—When, in a certain *p*H-range, the ratio of native to denatured molecules is *p*H-dependent, denaturation must be accompanied by a change in the average number of hydrogen ions dissociated from the protein molecules. Using equation V, A11 of the preceding paper,[4] we have

$$\frac{1}{RT}\frac{d\ \Delta F^0_H}{d\ln H} = \frac{1}{RT}\left(\frac{d\ \Delta F^0_h}{d\ln H}\right)_D - \frac{1}{RT}\left(\frac{d\ \Delta F^0_h}{d\ln H}\right)_N = r_D - r_N \quad (A10)$$

Converting the standard free energy to an equilibrium constant, we obtain

$$d\ln K_{obsd.}/d\ln H = r_N - r_D \quad (A11)$$

Since

$$K_{obsd.} = y/(1-y) \quad (A12)$$

according to eq. V3, we can write

$$dy/dpH = -2.303\ dy/d\ln H = -2.303\ (dy/d\ln K_{obsd.})(d\ln K_{obsd.}/d\ln H) = 2.303\ y(1-y)(r_D - r_N) \quad (A13)$$

Thus the rate of change of the fraction of the molecules in the denatured state with *p*H is directly proportional to the difference in proton binding between native and denatured molecules. It should be pointed out that equation A13 is quite general, *i.e.*, no assumption regarding special models has been made in deriving it.

[CONTRIBUTION FROM THE DEPARTMENT OF ORGANIC CHEMISTRY, UNIVERSITY OF ATHENS, ATHENS, GREECE, AND THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE, U. S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE, BETHESDA, MD.]

Studies on Arginine Peptides. III. On the Structure of Tricarbobenzoxy-L-arginine[1]

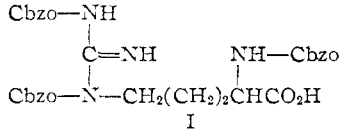
BY LEONIDAS ZERVAS, MILTON WINITZ AND JESSE P. GREENSTEIN[2]

RECEIVED FEBRUARY 10, 1961

The structural elucidation of N$^\alpha$,N$^\omega$,N$^\omega$-tricarbobenzoxy-L-arginine has been achieved. Thus, conversion of this compound to the *p*-nitrobenzyl ester, followed by the treatment with propionic anhydride, led to N$^\alpha$,N$^\omega$,N$^\omega$-tricarbobenzoxy-N$^\omega$-propionyl-L-arginine *p*-nitrobenzyl ester. Catalytic hydrogenolysis of the latter yielded N$^\omega$-propionyl-L-arginine which, when subjected to the action of acetic anhydride, was converted to N$^\alpha$,N$^\omega$-diacetyl-N$^\omega$-propionylanhydroarginine. Cleavage of the latter with water led to *N*-acetyl-*N'*-propionylurea and DL-α-acetylaminopiperidone which, upon identification, established the structure of the starting material. The relation of the structure of tricarbobenzoxyarginine to its utility as a peptide intermediate is considered.

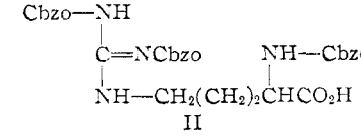
Introduction

Previous studies in this series[3,4] were concerned with the preparation of N$^\alpha$,N$^\omega$,N$^\omega$-tricarbobenzoxy-L-arginine and the utility of this and related compounds as intermediates in the synthesis of N- and C-terminal arginine peptides. The triacylated arginine derivative was secured readily by treatment of L-arginine with an excess of carbobenzoxy chloride in a highly alkaline aqueous medium and behaved, in its reactions, as a pure and discrete chemical entity. However, its exact structural assignment posed a somewhat puzzling problem, for although the position of one of the carbobenzoxy substituents on the α-nitrogen atom could be established with certainty, the positions of the other two carbobenzoxy groups on the nitrogen atoms of the guanidino nucleus permitted the assignment of one or the other of two equally plausible structures, as represented by formulas I and II, respectively. It is evidence leading to the



I

unequivocal designation of I as the correct structure with which the present communication is primarily concerned.[5]

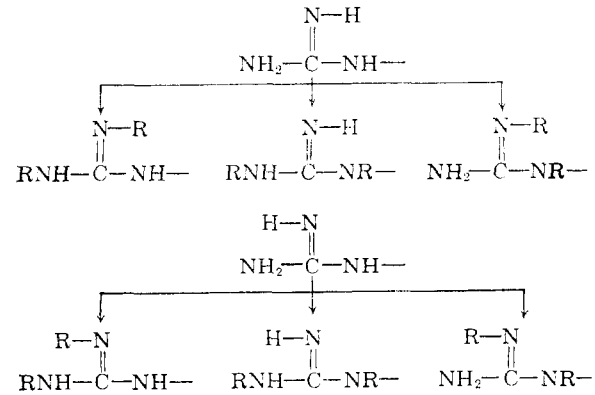


II

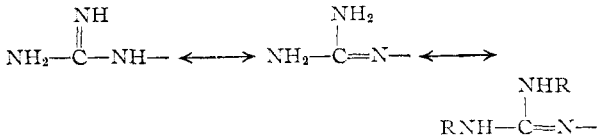
Results and Discussion

In an earlier communication,[3b,4b] the preferential cleavage of one of the two N$^\omega$-carbobenzoxy sub-

(5) Examination of the structure of the guanidino group indicates that diacylation of this moiety may, theoretically at least, lead to a number of *cis-trans* isomers, as in what follows



In addition, resonance may lead to the possibility



The present study is directed toward demonstrating the positions of the two acyl substituents on the nitrogen atoms of the guanidino moiety, leaving the stereochemical aspects of the problem for future consideration.

(1) A summary of this paper was presented at the 2nd European Peptide Symposium, Munich, September, 1959.

(2) Deceased February 12, 1959.

(3) (a) L. Zervas, M. Winitz and J. P. Greenstein, *Arch. Biochem. Biophys.*, **65**, 573 (1956); (b) L. Zervas, M. Winitz and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).

(4) (a) L. Zervas, T. Otani, M. Winitz and J. P. Greenstein, *Arch. Biochem. Biophys.*, **75**, 290 (1958); (b) L. Zervas, T. Otani, M. Winitz and J. P. Greenstein, *J. Am. Chem. Soc.*, **81**, 2873 (1959).

stituents of $N^{\alpha},N^{\omega},N^{\omega}$-tricarbobenzoxy-L-arginine by the action of methanolic alkali was described. That the N^{α},N^{ω}-dicarbobenzoxy-L-arginine formed thereby possessed the structure depicted by formula III ($R^1 = R^2 = C_6H_5CH_2OCO-$) was indicated both by the facile intramolecular cyclization of this material to N^{α},N^{ω}-dicarbobenzoxyanhydro-L-arginine (IV, $R^1 = R^2 = C_6H_5CH_2OCO-$) after treatment with such condensing agents as phosphorus pentachloride or dicyclohexylcarbodiimide[6] and by the subsequent degradation of IV to N-acetyl-N′-carbobenzoxyurea (VI, $R^2 = C_6H_5CH_2OCO-$, $R^3 = CH_3CO-$) and N^{α}-carbobenzoxyaminopiperidone (VII, $R^1 = C_6H_5CH_2OCO-$) by treatment with acetic anhydride, followed by the action of water on the intermediate (V, $R^1 = R^2 = C_6H_5CH_2OCO-$, $R^3 = CH_3CO-$) so derived. Although the structure of N^{α},N^{ω}-dicarbobenzoxy-L-arginine of itself afforded no clue as to whether the structure of $N^{\alpha},N^{\omega},N^{\omega}$-tricarbobenzoxy-arginine was most appropriately represented by formula I or formula II, (a) its facile conversion to the parent tricarbobenzoxy derivative upon interaction with carbobenzoxy chloride in aqueous alkali,[3a,b] when compared with the generally more drastic treatment required for acylation of the >C=NH group, (b) its ready conversion to the corresponding anhydro intermediate (IV, $R^1 = R^2 = C_6H_5CH_2OCO-$) in the presence of suitable condensing agents, (c) and the fact that all attempts to *isolate* the analogous tricarbobenzoxyanhydroarginine have hitherto proven unsuccessful,[4b] provided presumptive but certainly not conclusive evidence in favor of formulation I. In this connection, it is worthy of note that the action of acetic acid–acetic anhydride on nitro-L-arginine (III, $R^1 = H$, $R^2 = NO_2$) led to the formation of N^{α}-acetylanhydronitro-DL-arginine (IV, $R^1 = CH_3CO-$, $R^2 = NO_2$),[7] whilst the action of boiling acetic anhydride on L-arginine resulted in triacetylanhydro-DL-arginine (V, $R^1 = R^2 = R^3 = CH_3CO-$).[8]

In the search for a means which would permit unequivocal demonstration of the structure of tricarbobenzoxyarginine, attention was directed to the triacetylanhydroarginine of Bergmann and Köster.[8] Examination of the structure of this compound suggested that the preparation of a tetraacylated arginine derivative would be feasible if the δ-nitrogen atom, which was bound in lactam linkage with the carboxyl group, could instead be implicated in amide linkage with an acyl substituent. For the purpose at hand, acylation of the "unacylated" nitrogen atom of $N^{\alpha},N^{\omega},N^{\omega}$-tricarbobenzoxy-L-arginine with a suitably constituted substituent would, in fact, serve as an effective marker of this particular nitrogen atom. Toward this end, tricarbobenzoxyarginine first was converted to its *p*-nitrobenzyl ester derivative VIII in order to obviate the possibility of interference by the carboxyl function in the subsequent acylation reaction. That a tetraacylated arginine derivative could indeed be prepared then was demonstrated when the action of propionic anhydride on VIII at 100° permitted the ultimate isolation of crystalline $N^{\alpha},N^{\omega},N^{\omega}$-tricarbobenzoxy-$N^{\omega}$-propionyl-L-arginine *p*-nitrobenzyl ester (IX). Palladium-catalyzed hydrogenolysis of the latter compound proceeded with the simultaneous scission of the *p*-nitrobenzyl group and the three carbobenzoxy substituents.[9] The resulting N^{ω}-propionyl-L-arginine (X), wherein the unacylated nitrogen atom of the parent tricarbobenzoxyarginine compound was now effectively tagged with a propionyl group, was finally subjected to the classical Bergmann–Köster degradation.[8] Thus, treatment of X with an excess of boiling acetic anhydride led to a triacylated anhydroarginine (V, $R^1 = R^2 = CH_3CO-$, $R^3 = C_2H_5CO-$), which was subjected to cleavage *in situ* by the action of water to yield DL-α-acetylaminopiperidone (VII, $R^1 = CH_3CO-$) and a diacylurea. Identification of the last-mentioned product as N-

(6) Previous studies[4] demonstrated that the dicyclohexylcarbodiimide-mediated condensation of N^{α},N^{ω}-dicarbobenzoxy-L-arginine with amino acid esters proceeded with the formation of the desired acylated dipeptide ester in addition to appreciable amounts of a second product characterized as N^{α},N^{ω}-dicarbobenzoxyanhydro-L-arginine. More recent studies have revealed that the latter material could be obtained in high yield either by the treatment of N^{α},N^{ω}-dicarbobenzoxy-L-arginine with phosphorus pentachloride in anhydrous chloroform solution, followed by neutralization of the resulting acid chloride hydrochloride of N^{α},N^{ω}-dicarbobenzoxy-L-arginine or by interaction of this same diacylamino acid with dicyclohexylcarbodiimide in dry dioxane.

(7) S. M. Birnbaum and J. P. Greenstein, *Arch. Biochem. Biophys.*, **39**, 108 (1952).

(8) M. Bergmann and H. Köster, *Z. physiol. Chem.*, **159**, 179 (1926).

(9) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

acetyl-N′-propionylurea (VI, $R^2 = CH_3CO-$, $R^3 = C_2H_5CO-$) both permitted the rejection of formula II as representing the structure of tricarbobenzoxyarginine and established, beyond doubt, the validity of the structure indicated by formula I.

In conclusion, it is worthy of note that the highly favorable characteristics exhibited by tricarbobenzoxyarginine as a precursor in the synthesis of N-terminal arginine peptides, as described previously,[3,4] are further evidenced in the present study. Thus, although it was possible to acylate this material with the formation of a tetraacylated arginine derivative, the conditions here required were much more drastic than those ordinarily encountered in synthetic peptide techniques. This observation, in addition to the fact that tricarbobenzoxyarginine cannot, by virtue of its structure, undergo intramolecular cyclization to a lactam, in contrast to this potential hazard with other starting materials,[10] markedly lessens the danger of formation of undesirable secondary products during peptide synthesis.

Experimental

I. Structural Elucidation of Tricarbobenzoxyarginine. N^{α},N^{ω}-Tricarbobenzoxy-L-arginine *p*-Nitrobenzyl Ester (VIII).—To a solution of 5.7 g. (0.01 mole) of tricarbobenzoxy-L-arginine[3,4] and 1.4 ml. (0.01 mole) of triethylamine in 25 ml. of ethyl acetate is added 1.7 g. (0.01 mole) of *p*-nitrobenzyl chloride. The mixture is refluxed over a steam-bath for 20 hr., after which time it is washed twice with water and concentrated to dryness under reduced pressure. The crystalline residue so secured is treated with a little ethanol and the concentration to dryness repeated. After an additional repetition of this latter process, the residual material is recrystallized from boiling ethanol. The crystalline precipitate (needles) is recovered by filtration in the amount of 5.5 g. (78% of theor.); m.p. 100–103°. Recrystallization from ethanol raises the melting point to 102–104°; $[\alpha]_D$ +2.4° (10.4% in dioxane).

Anal. Calcd. for $C_{37}H_{37}N_5O_{10}$: C, 62.4; H, 5.2; N, 9.8. Found: C, 62.3; H, 5.3; N, 9.7.

N^{α},N^{ω}-Tricarbobenzoxy-N^{ω}-propionyl-L-arginine-*p*-Nitrobenzyl Ester (IX).—A solution of 2.8 g. (0.004 mole) of tricarbobenzoxy-L-arginine *p*-nitrobenzyl ester (VIII) in 12 ml. of propionic anhydride is heated at 100° for 30 min., the reaction mixture cooled to room temperature and 50 ml. of water added thereto. The mixture is stirred periodically until the excess of anhydride is destroyed and the precipitate which separates recovered by filtration and recrystallized, while still moist, from boiling ethanol. The crystalline product (needles), obtained in the amount of 1.8 g. (60% of theor.), melts at 83–84°. Another recrystallization from ethanol raises the melting point to 85–86°; $[\alpha]_D$ +1.7° (11% in dioxane).

Anal. Calcd. for $C_{40}H_{41}N_5O_{11}$: C, 62.6; H, 5.4; N, 9.1. Found: C, 62.7; H, 5.5; N, 9.1.

N^{ω}-Propionyl-L-arginine (X).—A solution of 3 g. (0.004 mole) of the above-described N^{ω}-propionyl derivative IX in methanol is treated with 1 ml. of acetic acid and the mixture subjected to palladium-catalyzed hydrogenolysis. Upon completion of the hydrogenolysis, a few milliliters of water is added in order to dissolve the small amount of material which separates during the reaction. The catalyst is removed by filtration and the filtrate concentrated under reduced pressure to a volume of about 15 ml. Treatment of the concentrate, first with acetone and then with ether, leads to the precipitation of 1 g. (83% of theor.) of N^{ω}-propionyl-L-arginine monoacetate as prisms; m.p. 149–151°. Recrystallization is effected from ethanol; m.p. 150–151°; $[\alpha]_D$ +1.2° (3.5% in water).

Anal. Calcd. for $C_{11}H_{22}N_4O_5$: C, 45.5; H, 7.6; N, 19.3. Found: C, 45.4; H, 7.8; N, 19.2.

(10) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1961, pp. 1068–1077.

N-Acetyl-N′-propionylurea (VI, $R^2 = CH_3CO-$, $R^3 = C_2H_5CO-$) and DL-α-Acetylaminopiperidone (VII, $R^1 = CH_3CO-$).—A mixture of 0.87 g. (0.003 mole) of N^{ω}-propionyl-L-arginine acetate in 5 ml. of acetic anhydride is boiled for 1–1.5 min. and the solution permitted to cool to room temperature. Water is then added thereto, with occasional stirring, in order both to destroy the excess acetic anhydride and cleave the N^{α},N^{ω}-diacetyl-N^{ω}-propionylanhydroarginine (V) arising from the action of acetic anhydride on X. The reaction mixture is concentrated to dryness under reduced pressure, the residue treated with a little water and the concentration to dryness repeated. The residual material is taken up in a small amount of water and the resulting solution permitted to concentrate to a volume of about 2 ml. by storage over sulfuric acid in a desiccator. After chilling at 4° for several hours, the crystalline precipitate of N-acetyl-N′-propionylurea is recovered by filtration and washed with a little water. (Save combined mother liquor and washings!) The yield is 0.33 g. (72% of theor.); m.p. 105°. Recrystallization is effected from water; m.p. 105–106°. An additional recrystallization from water, and then one from ethanol, raises the melting point to 108–109°.

For comparison purposes, N-acetyl-N′-propionyl-urea was synthesized. To a suspension of 5 g. (0.05 mole) of N-acetylurea in 10 ml. of propionic anhydride was added 0.1 ml. of concd. sulfuric acid. The mixture was heated for 20 min. at 100° with occasional shaking and then stored at room temperature for 48 hr., during which time some of the desired product crystallizes from solution. After the addition of ether, the crystals are filtered off; yield 5.6 g.; m.p. 107–108°. Two recrystallizations from water and one from ethanol raise the melting point to 109–110°. The mixed melting point of this material with that of the product from the degradation described above is 109–110°.

Anal. Calcd. for $C_6H_{10}N_2O_3$: C, 45.6; H, 6.4; N, 17.7. Found: C, 45.7; H, 6.5; N, 17.6.

The combined mother liquor and washings from the above process is concentrated to dryness by desiccation over sulfuric acid. A crystalline residue is procured which is dissolved in chloroform, the resulting solution is filtered to remove a small amount of insoluble material and the filtrate is treated slowly with ether. The precipitate of DL-α-acetylaminopiperidone is recovered by filtration; yield 0.3 g. (63%); m.p. 186–187°. Recrystallization from chloroform–ether raises the melting point to 188°.[11]

II. Preparation of N^{α},N^{ω}-Dicarbobenzoxyanhydro-L-arginine. Method A.—A suspension of 17.7 g. of N^{α},N^{ω}-dicarbobenzoxy-L-arginine[3,4b] in 60 ml. of anhydrous chloroform is cooled to about −10°, and 9.2 g. of phosphorus pentachloride is added thereto. The mixture is shaken at −10° until almost all of the phosphorus pentachloride has disappeared and is then treated with several volumes of pre-cooled petroleum ether. The resulting precipitate of the chloride hydrochloride derivative of N^{α},N^{ω}-dicarbobenzoxy-L-arginine is recovered by filtration, washed thrice with cold petroleum ether and then dissolved with shaking in 100 ml. of cold chloroform to which 20 ml. of anhydrous triethylamine had been added. After storage for 30 minutes at room temperature, the chloroform solution is washed thrice with water, once with 5% potassium carbonate, twice with water and finally once with water containing a few drops of acetic acid. The chloroform solution is evaporated to dryness, a few ml. of absolute ethanol added to the residue and the evaporation repeated. Following treatment of the residual material with ethanol and storage of the resulting solution for several hours in the cold, a crystalline material separates which is recovered by filtration, washed with a little ethanol, shaken in about 100 ml. of 5% potassium carbonate solution for 15 minutes, filtered, washed with water and dried; yield 10.5 g.; m.p. 143–144°. Recrystallization is effected from ethanol; yield 7.0 g.; m.p. 147°; $[\alpha]^{25}_D$ −12.4° (4.0% in chloroform).

Anal. Calcd. for $C_{22}H_{24}N_4O_5$: C, 62.2; H, 5.7; N, 13.2. Found: C, 62.3; H, 5.9; N, 13.2.

Method B.—A solution of 0.17 g. of anhydrous *p*-toluenesulfonic acid in a few milliliters of anhydrous dioxane is concentrated to dryness in order to remove traces of water. The residue is dissolved in 40 ml. of dry dioxane and treated

(11) A melting point of 187–188° has been reported[9] for this compound.

with 4.4 g. of N^{α},N^{ω}-dicarbobenzoxy-L-arginine and 2.2 g. of N,N-dicyclohexylcarbodiimide.[12] After the resulting suspension has been stirred at room temperature for about 8 hr., 1.5 ml. of water is added and the stirring continued for an additional 30 minutes. The white precipitate of dicyclohexylurea (2.2 g.) is removed by filtration, the filtrate evaporated to dryness and the residue treated with ethanol and evaporated to dryness. Upon trituration of the residual material with a small amount of cold ethanol, 3.6 g. of the product is obtained; m.p. 146–147°, unchanged by mixed m.p. with the material prepared by method A above; $[\alpha]^{25}D$ −12.2° (4% in chloroform).

Method C.—Four and four-tenths grams of dry, finely pulverized N^{α},N^{ω}-dicarbobenzoxy-L-arginine is dissolved in a mixture of 40 ml. of anhydrous chloroform and 1.4 ml. of anhydrous triethylamine, with shaking and occasional heating at 40–50°. The resulting solution is cooled to 0°, treated with 1.0 ml. of ethyl chloroformate and after storage at 0° for 5 min., treated with 1.4 ml. of triethylamine.[13] After additional storage for 30 min. at room temperature, the reaction mixture is washed twice with water and twice with dilute acetic acid, dried over anhydrous sodium sulfate and concentrated to dryness. Removal of the final traces of chloroform is achieved by the addition of a small amount of ethanol to the residue followed by a further concentration to dryness. The crystalline residue is triturated with a small amount of ethanol and filtered over suction; yield 3.6 g. After recrystallization from ethanol, the yield is 3.0 g.; m.p. 146–147°, unchanged by mixed m.p. with the products obtained by methods A and B above; $[\alpha]^{25}D$ −12.3° (4% in chloroform).

(12) The *p*-toluenesulfonic acid is here employed to form a salt with the N^{α},N^{ω}-dicarbobenzoxyarginine and thus not only permit the solution of this latter material in dioxane, wherein it is generally insoluble, but also facilitate its reaction with the dicyclohexylcarbodiimide reagent.

(13) As the addition of ethyl glycinate *in lieu* of triethylamine leads to the same yield of N^{α},N^{ω}-dicarbobenzoxyanhydroarginine, in the absence of detectable acyldipeptide ethyl ester formation, it may be concluded that the mixed anhydride formed by the interaction of ethyl chloroformate with N^{α},N^{ω}-dicarbobenzoxyarginine undergoes immediate intramolecular reaction with the nitrogen atom of the guanidino moiety.

[CONTRIBUTION FROM THE VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA]

Studies on the Amino Acid Sequence of Tobacco Mosaic Virus (TMV) Protein. IV. The Amino Acid Sequences of an Eicosapeptide and a Heptadecapeptide Isolated from a Tryptic Digest of TMV Protein[1]

BY DUANE T. GISH[2]

RECEIVED FEBRUARY 10, 1961

The amino acid sequence of an eicosapeptide, ileu·ileu·gluNH$_2$·val·glu·aspNH$_2$·gluNH$_2$·ala·aspNH$_2$·pro·thr·thr·ala·glu·thr·leu·asp·ala·thr·arg, representing positions 93 through 112 of TMV protein, and the amino acid sequence of a heptadecapeptide, ser·ser·phe·glu·ser·ser·ser·gly·leu·val·try·thr·ser·gly·pro·ala·thr, representing the C-terminal portion, or positions 142 through 158, of TMV protein, have been determined.

Previous papers from this Laboratory[3-9] have reported the results of amino acid sequence studies on TMV protein. Based upon this work and, as yet, unpublished work, plus a few sequences tentatively accepted from the literature,[10] a complete amino acid sequence for TMV protein was proposed.[11] Anderer, *et al.*,[10] have proposed a nearly complete structure for TMV protein. At the time of the publication of their paper, Anderer and coworkers had not established the question of the amidation of 18 of the aspartic and glutamic acid residues and had not established the sequences of the amino acids occupying positions 26 and 27 nor those occupying positions 98 through 102 of the protein chain. Work was proceeding concurrently in Berkeley, and we wish to report the sequence of an eicosapeptide, the amino acids of which occupy positions 93 through 112 of the protein, and the sequence of a heptadecapeptide, the amino acids of which occupy positions 142 through 158, or the C-terminal portion of the protein. These peptides were isolated from a tryptic digest of the protein. The amino acid sequence of the C-terminal peptide has in part been previously published[8] and *in toto* referred to[12] and has been confirmed by Anderer, *et al.*[10]

The eicosapeptide, designated DCA-I-3 or Peptide 8 in a previous paper,[11] will be referred to in this paper as Peptide 8, and the heptadecapeptide, referred to in previous papers as the C-terminal peptide,[8,12] will be referred to as Peptide 12.

Peptides 8 and 12 were found in the fraction insoluble in the 2-butanol–0.1 *M* dichloroacetic acid system used for the countercurrent distribution of the tryptic digest of TMV protein.[3] These two components were separated by countercurrent distribution in the system 1-butanol–pyridine–0.1% acetic acid (5:3.5:12).[13] The distribution pattern after 1000 transfers is shown in Fig. 1. Although these peptides could not be recovered quantitatively from a tryptic digest of the protein due to some coprecipitation during the isolation of Peptide 1, or "I"-peptide,[3] and to the fact that Peptide 12 contains a tryptophyl–threonine bond very sensitive to traces of chymotryptic activity in the trypsin, they were recovered in quite sub-

(1) This paper has been aided by a U. S. Public Health Service Grant.

(2) The Upjohn Co., Kalamazoo, Michigan.

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